The evolution of bacterial mutation rates under simultaneous selection by interspecific and social parasitism

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Many bacterial populations harbour substantial numbers of hypermutable bacteria, in spite of hypermutation being associated with deleterious mutations. One reason for the persistence of hypermutators is the provision of novel mutations, enabling rapid adaptation to continually changing environments, for example coevolving virulent parasites. However, hypermutation also increases the rate at which intraspecific parasites (social cheats) are generated. Interspecific and intraspecific parasitism are therefore likely to impose conflicting selection pressure on mutation rate. Here, we combine theory and experiments to investigate how simultaneous selection from inter- and intraspecific parasitism affects the evolution of bacterial mutation rates in the plant-colonizing bacterium Pseudomonas fluorescens.

Both our theoretical and experimental results suggest that phage presence increases and selection for public goods cooperation (the production of iron-scavenging siderophores) decreases selection for mutator bacteria. Moreover, phages imposed a much greater growth cost than social cheating, and when both selection pressures were imposed simultaneously, selection for cooperation did not affect mutation rate evolution. Given the ubiquity of infectious phages in the natural environment and clinical infections, our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution.

1. Introduction

One hypothesis for the evolution of sexual reproduction is an increased ability to evade parasites: the ‘Parasite Red Queen’ hypothesis [1–8]. Parasites become adapted to the most frequent host genotypes, and host sex can result in novel genotypes that escape infection by the most common parasites. However, parasites can quickly adapt, and so there is continual selection for genotypic diversity in the presence of parasites. However, another form of parasitism, social parasitism, may itself be a cost of sexual reproduction. Specifically, the genetic diversity produced by sex means that interacting individuals are less likely to share the same alleles for cooperation. This reduction in relatedness reduces the indirect benefits required to select for cooperative behaviour. This idea that ‘sex is an antisocial force in evolution’ was first proposed by Wilson [9], and has been explored both theoretically and comparatively [10–14], demonstrating that highly cooperative societies such as those of the eusocial insects or cooperative breeders are likely to be preceded by strict lifetime monogamy (rather than promiscuity), whereby relatedness between interacting individuals is predictably high. Sexual reproduction can therefore create a trade-off between resistance to inter- and intraspecific parasitism [15,16].

The evolution of mutation rates in obligatory asexual species may be affected by similar conflicting selection pressures from inter- and intraspecific parasitism. Many bacterial populations harbour hypermutable bacterial strains (10- to 1000-fold increase in genomic mutation rate [17–20]; with the frequency of hypermutators sometimes in excess of 30% [21] in clinical infections. Hypermutators increase in frequency through hitchhiking with beneficial mutations, hence continual changing selection pressures tend to result in increases in
2. Theoretical model and analysis

We develop a simple mathematical model to make qualitative predictions about the evolution of bacterial mutation rates $\mu$ in the presence of social cheats and parasites. We assume that higher mutation rates both increase bacterial resistance to parasites and the local abundance of social cheats. We consider a metapopulation composed of a large number of patches, each of which is colonized by a single bacterial strain with mutation rate $\mu$, with $0 \leq \mu \leq 1$. All strains are assumed to be functional for the cooperative trait, and cheats arise by loss-of-function mutations. While cheats impose a growth cost on cooperative lineages, we assume that cheats are poor colonizers of new patches, and therefore evolutionarily unviable. We assume that the fitness $w$ of a bacterial strain with mutation rate $\mu$ is a function of its survival $S$ and its growth $G$, such that $w(\mu) = S G$.

Bacterial survival decreases as parasite density $\pi$ increases, while it increases as bacterial hosts evolve resistance to parasites. For simplicity of analysis, we assume that the probability that bacterial hosts acquire resistance alleles is equal to their mutation rate $\mu$, and that parasite-induced mortality decreases linearly with resistance. As a result, the survival cost imposed by parasites on their bacterial hosts is $(1-\mu)\pi$, such that the survival of a strain with mutation rate $\mu$ is $S = 1 - (1-\mu)\pi$. Note that in the absence of parasites ($\pi = 0$), or when there is full resistance ($\mu = 1$), survival is assumed to be 1.

We assume that the costs associated with social cheats depend on three factors: (i) the local abundance of cheats; (ii) the intrinsic cost of cheats to cooperators and (iii) parasite negative density-dependent regulation of hosts’ cooperative growth. We assume that the local abundance of cheats is equal to the mutation rate $\mu$ of the local bacterial strain. Cheats are assumed to impose an intrinsic cost $c$ on cooperative growth. In addition, cooperative growth may be subjected to density-dependent regulation by parasites, which reduces the impact of cheats on cooperative growth.

Our objective is to determine the evolutionary stable (ES) mutation rate $\mu^*$. We find that the marginal fitness benefit of a slight increase in the mutation rate is given by $B(\mu) = \pi(1 - (1-\alpha\pi)c)\mu$ and the corresponding cost is $S(1-\alpha\pi)c$. Thus, as long as there is some growth, the marginal fitness cost of a slight increase in the mutation rate is given by $C(\mu) = (1-\pi(1-\mu))\pi(1 - (1-\alpha\pi)c)\mu = S(1-\alpha\pi)c$. Thus, as long as there is some survival, the growth cost is low if (i) there is strong parasite density-dependent regulation (high $\alpha\pi$) and (ii) the intrinsic cost of cheats is low (low $c$). The ES mutation rate is the value $\mu^*$ for which the marginal cost exactly cancels the marginal benefit of a slight increase in the mutation rate, i.e. $B(\mu^*) = C(\mu^*)$. This is given by

$$\mu^* = \frac{1}{2} \left( \frac{1}{\pi(1-\alpha\pi)} - \frac{1-\pi}{\pi} \right).$$

We find that the ES mutation rate $\mu^*$ decreases with increasing intrinsic cost of cheats ($\partial \mu^*/\partial c < 0$). By contrast, the ES mutation rate $\mu^*$ increases with increasing degree of parasite negative density-dependent regulation of hosts ($\partial \mu^*/\partial \alpha > 0$) and with increasing parasite density ($\partial \mu^*/\partial \pi > 0$; figure 1).
3. Experimental material and methods

(a) Strains

The P. fluorescens strain SBW25 [39] was used as a wild-type, and the strain SBW25mutL, that has a mutation in the mismatch repair gene mutL, was used as a hypermutator. SBW25mutL exhibits a spontaneous mutation rate 100 times higher than that of SBW25 and is resistant to the antibiotic gentamicin [24], allowing it to be distinguished from the wild-type. We used the lytic bacteriophage phi 2 (42) [33].

(b) Experimental design

To ensure that each population was colonized by a single strain (i.e. relatedness = 1) at the first transfer into medium, each bacterial culture was grown in 6 ml King’s Medium B (KB; 10 g glycerol, 20 g proteose peptone no. 3, 1.5 g K₂HPO₄·3H₂O, 1.5 g MgSO₄·7H₂O, per litre), for 24 h at 27°C, after which it was diluted with M9 buffer and grown for 36 h on KB agar at 27°C. A single colony could then be selected and further grown in KB medium at 27°C for 24 h. This single-strain culture could then be used to establish populations.

We manipulated phage and iron availability in a fully factorial experiment, setting up each of our four treatments (i) iron + phage, (ii) iron only, (iii) phage only and (iv) no iron, no phage) in 96-well plates. We used six replicates for each treatment, with each replicate consisting of a meta-population made up of six patches (wells). Each well was inoculated with 180 µl KB medium, with three wells of each replicate initially inoculated with approximately 10⁷ colony-forming units (CFUs) of clonal wild-type bacteria, and three wells inoculated with approximately 10⁷ CFUs of clonal mutator bacteria. Iron limitation was created by the addition of 100 µg ml⁻¹ human apotransferrin and 20 mM sodium hydrogen carbonate (NaHCO₃) to standard KB medium immediately before use [35]; NaHCO₃ was also added to non-iron-limited treatments. Approximately 10³ phages were added to phage treatments in 10 µl KB and the same volume of KB media added to non-phage wells. Plates were incubated at 27°C under static conditions.

Every second day, aliquots (2 µl) from all four treatments were transferred to new KB medium (± Fe) using a sterile pin replicator. For every four transfers (8 days), 100 µl from each of the six wells within a replicate was mixed, simulating global competition [36] and plated out on KB and KB + gentamicin agar to assess frequencies of mutator and wild-type bacteria (see below). Phage was isolated from this mixture each time, by adding 10% chloroform and centrifuging at 13 000 r.p.m., which lysed and pelleted bacteria. Six colonies from each KB agar plate were randomly selected, grown independently for 24 h in 180 µl KB medium at 27°C and each was subsequently used to inoculate a new well within a treatment (10⁷ CFUs), to ensure high relatedness, and hence the potential for selection for cooperation was continually re-established. For phage treatments, both sympatric (10 µl) phages and ancestral (10 µl) phages were added (to ensure continual selection for phage resistance where sympatric phages had reached low densities); equivalent volumes of phage-free KB media were added to no-phage treatments. Mixing and plating were performed five times in total, for approximately 250 generations of bacterial growth (carrying out six transfers instead of four between mixing after the third assay).

(c) Assays

The relative fitness of mutator and wild-type strains was assessed by plating a mixture of all patches within a replicate onto both KB agar (for an estimate of total density) and KB agar supplemented with 10 µg ml⁻¹ gentamicin (on which only gentamicin-resistant mutator bacteria can grow) over 36 h, resulting in a KB and KB + gentamicin plate for each replicate and six plates of each type per treatment. Colony types were also categorized by colour into either siderophore producers (yellow/green) or non-producers (white), based on the fact that the primary siderophore of P. fluorescens, pyoverdine, is yellow/green. As the amount of colony pigmentation varied considerably, we classified a colony showing any yellow/green pigmentation after 36 h as a producer [26]. We carried out an additional experiment to verify that the appearance of milky white colonies indeed represented the evolution of siderophore negative cheats. We selected 19 wild-type and 19 milky colonies at random at the final timepoint, and inoculated each colony into iron-limited KB media. Populations were grown at 27°C for 24 h, after which, fluorescence of 180 µl of culture was measured at 460 nm, following excitation at

Figure 1. (a,b) The ES mutation rate (µ*) as a function of parasite density (π) and the intrinsic cost of cheats (c), for various levels of parasite density-dependent regulation of hosts (α). If there is no parasite density-dependent regulation (α = 0), then when parasite density is low and the cost of cheats is high, selection favours low mutation rates (c ≥ π/(1 + π); µ* = 0). If parasite density is equal to the intrinsic cost of cheats, then intermediate levels of mutation rates are favoured (π = c, µ* = 0.5; dashed white line). If parasite density is high relative to the cost of cheats, then selection favours high mutation rates (c ≤ π/(1 − π); µ* = 1). If there is strong parasite density-dependent regulation (α = 1), the ES mutation rate becomes less sensitive to changes in the cost of cheats (c).
400 nm using a Biotek Synergy 2 spectrophotometer (standard protocol for measuring pyoverdine production [40,41]). Milky colonies produced pyoverdine at significantly lower levels than wild-type (Student’s t-test, $T_{18.017} = 15.7631$, $p < 0.0001$), and so any milky colonies that emerged during the experiment were labelled siderophore cheats.

(d) Costs of cheating and phage parasitism
To directly assess the relative cost of cheats and parasites on the growth of wild-type bacteria, wild-type SBW25 and an isogenic cheat strain (SBW25ΔpvdL), with the primary siderophore, pyoverdine, knocked out, [42]) were grown in isolation type (Student’s t-test, $T_{8} = 4.6885$, $p < 0.001$), however, in the presence of phages, this relationship was reversed and cheats grew significantly more than wild-type (Student’s t-test, $T_{11.124} = 3.3046$, $p < 0.01$). The reduction in the density of wild-type bacteria brought about by cheats was significantly smaller than the reduction caused by the presence of phages (Student’s t-test, $T_{6.872} = 4.5904$, $p < 0.01$). Based on our simple model predictions, these ecological results suggest that phage-imposed selection for mutators is likely to be greater than that imposed by cheats. A competition experiment between wild-type SBW25 and SBW25ΔpvdL verified that cheats can invade cooperators under iron-limited conditions (Student’s t-test (alt = 1), $T_{5} = 2.5039$, $p = 0.05$), despite the poorer performance of cheats compared with cooperators as monocultures; i.e. pyoverdin production is an altruistic trait.

4. Experimental results

(a) Costs of cheats and phages
To establish the relative magnitude of costs of cheating and phage parasitism on bacterial growth, we measured the densities of siderophore cheat and wild-type strains grown as monocultures in the presence and absence of ancestral bacteriophages after 24 h growth under iron-limited conditions. Phages caused a significant reduction in the density of wild-type (Student’s t-test, $T_{7.515} = 5.6787$, $p < 0.001$) and cheat phenotypes (Student’s t-test, $T_{11.542} = 2.4959$, $p < 0.05$). Consistent with previous work [35,42], cheats reached significantly lower densities than wild-type bacteria in the absence of phages (Student’s t-test, $T_{8.609} = 4.6885$, $p < 0.001$), however, in the presence of phages, this relationship was reversed and cheats grew significantly more than wild-type (Student’s t-test, $T_{11.124} = 3.3046$, $p < 0.01$). The reduction in the density of wild-type bacteria brought about by cheats was significantly smaller than the reduction caused by the presence of phages (Student’s t-test, $T_{6.872} = 4.5904$, $p < 0.01$). Based on our simple model predictions, these ecological results suggest that phage-imposed selection for mutators is likely to be greater than that imposed by cheats. A competition experiment between wild-type SBW25 and SBW25ΔpvdL verified that cheats can invade cooperators under iron-limited conditions (Student’s t-test (alt = 1), $T_{5} = 2.5039$, $p = 0.05$), despite the poorer performance of cheats compared with cooperators as monocultures; i.e. pyoverdin production is an altruistic trait.

(b) Competition between mutators and wild-type over evolutionary time scales
We predicted that phages should select for higher mutation rates, while selection for cooperation should select against cheats. Phage-imposed selection was manipulated by the presence versus absence of phages, whereas selection for siderophore-mediated cooperation was manipulated by changing iron availability (siderophores are required when iron is limited) in experimental metapopulations. While the frequency of mutators increased through time in the presence of phages and decreased in the absence of phages (LMER: non-significant phage interaction, $\chi_{1,5}^{2} = 9.3343$, $p < 0.01$; figure 2) iron availability had no impact on the frequency of mutators (LMER: $\chi_{1,5}^{2} = 0.3097$, $p = 0.58$). Moreover, there was no interaction between the presence of phage and iron availability (LMER: non-significant phage × interaction, $\chi_{1,5}^{2} = 0.187$, $p = 0.67$). Selection for mutators via the presence of external viruses, therefore was not affected by any conflicting selection for cooperation by iron limitation.

(e) Statistical analysis
R software [43] was employed for all statistical analyses. Maximum-likelihood analysis was carried out using Linear Mixed-Effects Revised models with arcsine square root (proportion of mutators/cheats) as a response variable, and iron, phage and time, including two-way interactions, as explanatory variables. A third model with cheat frequency as the response variable was employed to investigate any affect mutator frequency might have on the appearance of cheats and the extent to which this was affected by iron limitation. Iron and phage presence/absence were fitted as factors and dryad.tq34k. Available from the dryad repository: doi.org/10.5061/dryad.tq34k.
The frequency of siderophore cheats increased through time in all treatments but significantly more so when there was selection for cooperation by limiting iron availability (LMER: timepoint × iron interaction $\chi^2_{1.5} = 3.9498$, $p < 0.05$; figure 3). Phage presence did promote the production of cheats through time, but this trend was not significant (LMER: non-significant phage × time interaction, $\chi^2_{1.7} = 2.1579$, $p = 0.14$). Furthermore, no interaction was observed between iron availability and the presence of phages in determining cheat frequency (LMER: non-significant phage × iron interaction $\chi^2_{1.8} = 0.6231$, $p = 0.43$). Cheat frequency increased with the frequency of mutators, but only in iron-rich populations (LMER: mutator frequency × iron interaction: $\chi^2_{1.5} = 8.1479$, $p < 0.01$).

5. Discussion

Here, we investigate both theoretically and empirically how selection imposed by interspecific parasites (bacteriophages) and intraspecific parasites (social cheats) affects the evolution of mutation rates in bacteria. Theoretically, selection for mutator bacteria decreases when there is selection for cooperation because mutators are more likely to generate social cheats, and increases in the presence of phage because mutators are more likely to generate phage resistance mutations. Our short-term growth rate experiments suggest that phages impose a much greater growth rate cost than social cheating in this experimental context. Moreover, the impact of phages on density was less for cheat than wild-type populations. Feeding this back into our simple model suggests that the strength of phage-imposed positive selection on mutators will be greater than cooperation-imposed negative selection on mutators. Our evolution experiment was consistent with this prediction: mutator frequency increased in the presence of phages but was not influenced by whether or not there was selection for cooperation.

That coevolution with phages favours mutator bacteria is consistent with previous work using this system [22,24,25]. Pal et al. [24] found that after approximately 170 bacterial generations, mutation rates had increased 10- to 100-fold in nine out of 36 populations in the presence of coevolving phages, whereas no significant change in mutation rate was observed in populations evolving in the absence of phages. Qualitatively identical results were observed when the wild-type and an isogenic mutator were competed at equal ratios in the presence and absence of phages. However, in these experiments bacteria were cultured as single populations. Our results reveal similar phage-imposed selection for mutation rates in high-relatedness metapopulations. Arguably, such metapopulation structure presents a more ecologically relevant scenario for bacteria, where microcolonies are founded by single bacteria, which then go extinct or go on to colonize new patches [44].

The lack of association between selection for siderophore-mediated cooperation and mutator frequency is initially surprising, as a previous study showed such a relationship using the related bacterium, *P. aeruginosa* [27]. Specifically, in an evolution experiment of a similar duration to this study (approx. 250 generations), mutator frequency was significantly lower in iron-limited metapopulations when patches were established with single colonies (high relatedness, hence strong selection for cooperation) compared with multiple colonies. In both studies, cheats of both species show reduced growth under iron-limited conditions (this study, [36,42,45]). The discrepancy between these results is likely to have arisen because (i) different species and media were used, and hence the cost of cheating may be different, (ii) selection for cooperation in this study was manipulated by iron availability as opposed to number of colonies founding a patch and (iii) we used the mutL mutator strain rather than mutS as in previous studies [24,27], and differences in genetic effects of the specific gene may account for discrepancies. However, studies in *Escherichia coli* suggest these mutations have very similar effects [46].

Our model and ecological experiment suggests a simple but potentially general interaction between social cheating and parasitism: parasites have less of an impact on reducing cheat population density, and hence the relative impact of cheating on population growth is reduced in the presence compared with the absence of parasites. This is a simple extension of the more general finding that enemies typically reduce high-density populations relatively more than low-density populations [47,48]. In addition to weakening selection against mutators, this reduced costs of cheats also suggests that cheats may be relatively favoured in the presence versus absence of parasites. By contrast, recent theory and experiments suggest cheating may be less favoured in the presence of parasites, because cheats are likely to be initially rare in newly colonized patches, and hence less likely to evolve resistance mutations [35,49]. Both mechanisms are not mutually exclusive, with the former relevant when cheats are at sufficiently high frequency to affect growth and the latter when cheats are initially rare. The simultaneous operation of both mechanisms may explain our finding that phages had no net effect on the presence of cheats.

High mutation rates can result in rapid resistance to antibiotics and bacteriophage, and clinical studies have shown hypermutators to be associated with poorer lung function in CF patients [50]. Therefore, it is crucial to develop an understanding of how different selection pressures involved in their persistence interact with each other.

![Figure 3](http://rspb.royalsocietypublishing.org/lookup/image/5cc3ce8c76e8f0b46234a8a3481c766f)

**Figure 3.** When iron is limited, cheats reach higher frequencies through time than when iron is present ($\chi^2_{1.5} = 3.9498$, $p < 0.05$). Data are plotted using predictions from minimal LMER model (cheat frequency ~ time : iron + 1/population).
the ubiquity of infectious phages in the natural environment [51] and clinical infections [52,53], our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution in natural environments.

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